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PRINCIPAL INVESTIGATOR: Jerry J. Buccafusco

CONTRACTING ORGANIZATION: Medical College of Georgia
Department of Pharmacology
and Toxicology
Augusta, Georgia 30912-2300



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Previous studies in this laboratory have demonstrated that the $\alpha_2$-adrenergic agonist clonidine can offer protection against the acute and chronic toxicity to soman in rats and mice. The purpose of this study was to determine whether addition of clonidine to a standard pretreatment protective regimen could offer added benefit. The standard regimen consisted of physostigmine salicylate (150 µg/kg) and atane (trihexyphenidyl hydrochloride, 2 mg/kg). Rats were assigned to the following groups: 1) saline controls; 2) saline, i.m., followed 30 min later by one of several doses (60-110 µg/kg) of soman, s.c.; 3) saline, i.m., followed 10 min later by the standard pretreatment regimen, i.m., followed 30 min later by one of several doses of soman (160-300 µg/kg), s.c.; and 4) clonidine hydrochloride (1 mg/kg), i.m., followed 10 min later by the pretreatment regimen, followed 30 min later by soman. All animals were examined acutely, and survivors were examined, over a 3-week period following soman administration. Addition of clonidine to the standard regimen did not enhance survival unless the clonidine was administered after the regimen. Clonidine addition to the standard regimen also was of benefit in reducing soman-induced tremor. Addition of clonidine appeared to hasten the return-to-normal motor behavior after soman; however, all groups exhibited normal motor behavior in 9 days. Despite apparent normal motor behavior, soman-treated animals exhibited a marked performance deficit in the passive-avoidance parameter 3 weeks after injection. The standard regimen partially reversed this effect; addition of clonidine to the standard regimen completely reversed the effect. These results indicate that clonidine provides a measure of protection against chronic behavioral deficits caused by soman intoxication. (continued)

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In the second portion of this study, we compared the ability of clonidine and a series of α-adrenergic receptor agonists added to a standard regimen of pyridostigmine to offer protection against soman toxicity. We found that addition of clonidine or one of the analogues produced more benefit than using pyridostigmine alone. Since pyridostigmine offers protection primarily against the peripheral manifestations of soman toxicity, the added benefit of clonidine is consistent with a central mode of action for the $\alpha_2$ agonist. Of the 5 compounds tested thus far, clonidine and guanfacine provided the best overall enhancement in protection when combined with pyridostigmine.

In the last portion of this study soman was employed in two species and under different conditions of acute, sub-acute and chronic administration. Soman was examined for its effects on regional brain muscarinic receptor regulation *in vivo* in synaptosomal fractions prepared *in vitro*. Acute administration of soman (s.c.) significantly reduced the density of receptors 2 hr after treatment as measured by [$^3$H]methylscopolamine binding in cortex and hindbrain, at a dose which inhibited 90% cholinesterase (ChE) activity in rats. The levels of muscarinic receptors (Bmax) returned to control values within 24 hrs. Pretreatment with 1 mg/kg of clonidine reversed the soman-induced muscarinic receptor down-regulation, confirming our earlier studies in rats. Acute administration of soman to guinea pigs at doses in the $LD_{50-90}$ range did not alter binding in the respective brain regions. Chronic treatment of rats with soman for 7 days (which resulted in 95% inhibition of ChE at the time receptor binding was measured) did not alter binding parameters in any of the brain regions. These results support the concept that brain ChE inhibition is not the only factor which determines whether down regulation will occur and that receptor regulation may be different in the various brain regions and in different species. The ability of clonidine to protect against soman-induced receptor down-regulation is most likely related to its ability to diminish acetylcholine release.

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# INTRODUCTION

In developing antidotes to poisoning by cholinesterase (ChE) inhibitors, several potential target sites at the cholinergic synapse have been studied, including the postsynaptic receptor and acetylcholinesterase (AchE) itself. Muscarinic receptor blocking agents such as atropine have been and continue to be the primary pharmacological intervention in cases of anticholinesterase poisoning. Oxime reactivators may prove useful when the enzyme is inhibited by an organophosphorus agent. One site which has received much less attention is the presynaptic site at the cholinergic nerve terminal. It is reasonable to expect that reducing acetylcholine release would decrease the toxicity of ChE inhibitors. In fact, inhibitory mechanisms are in place presynaptically to reduce cholinergic neuronal function in situations of postsynaptic overstimulation. These mechanisms include down-regulation or decreased postsynaptic receptor numbers, and decreased release of transmitter from the cholinergic nerve terminal. In cases where poisoning is slow enough, such adaptive changes allow for significant degrees of ChE inhibition without toxicity and even without overt symptoms. In cases of acute severe poisoning, such adaptive mechanisms are too slow to prevent the development of toxicity. Acceleration of presynaptic down-regulation by pharmacologic agents, therefore, may be of use under such circumstances. The examination of this approach to protection has been limited, perhaps due to a paucity of presynaptic cholinergic blocking agents, or from the fear that such agents might prove highly toxic. Complete blockade of acetylcholine release with botulinum toxin underscores this concern. However, an agent which merely accelerates presynaptic down-regulation without completely inhibiting transmitter release might be of value.

In support of this possibility our experiments [4] first demonstrated a marked protection by clonidine against the manifestations of physostigmine toxicity. In the mouse, clonidine's protective actions were associated with significant inhibition of the increase in brain acetylcholine induced by the reversible ChE inhibitor. That the mechanism of protection was primarily through central cholinergic and peripheral muscarinic pathways was indicated by the lack of protection afforded by clonidine against the toxic effects of the selective, peripherally acting ChE inhibitor neostigmine. More recent studies employing organophosphate ChE inhibitors (soman and echothiophate) substantiated the physostigmine studies [1,7,8]. Moreover, the combined use of atropine and clonidine in the pretreatment regimen was found to enhance survival following soman administration. During these experiments it was consistently noted that clonidine-pretreated mice which survived $LD_{50}$ doses of soman had fewer behavioral side effects than mice which did not receive clonidine. This observation was confirmed in a rat model in which the toxic behavioral effects induced by soman administration were quantitated [10]. Again clonidine offered protection against the lethal as well as the toxic behavioral effects of soman. This behavioral toxicity included the development of tremor, hindlimb extension, convulsions and jerking motions, chewing, and excessive salivation. Soman also decreased the expression of normal ongoing behaviors such as sniffing, rearing, and general locomotor activity. The ability of clonidine to inhibit soman-induced convulsive behavior [10,12] is consistent with its anticonvulsive activity in other animal models [see 3], a feature of its protection which might help to limit the development of more permanent toxic manifestations. The protective effects of clonidine and atropine were usually synergistic, even though clonidine antagonized some of the stereotyped behaviors elicited by protective doses of atropine [37]. Thus, while enhancing the protective actions of atropine, clonidine also may reduce atropine-induced side effects. The mechanism for this latter effect is yet to be identified.

The mechanism of the protective actions of clonidine has been investigated and appears to be more complex than simply the inhibition of acetylcholine release. That is, while clonidine does produce a marked inhibition of acetylcholine synthesis and release at peripheral and central muscarinic synapses, its other actions on the cholinergic system include a reversible inhibition of AChE and a reversible inhibition

of muscarinic receptors [1,7,9]. This interaction with the enzyme was observed both *in vivo* and *in vitro* preparations, and, in both cases the permanent inhibition of enzyme activity produced by soman was reduced by clonidine treatment. This mode of protection of the enzyme may be similar to that produced by reversible carbamate ChE inhibitors, such as pyridostigmine. Reversible inhibition of cholinesterase essentially protects the enzyme from permanent inactivation by irreversible agents such as soman. Clonidine and many of the tested analog were also found to interact directly with muscarinic receptors, in an atropine-like manner. Therefore, clonidine and several analog afford protection against soman poisoning by at least three mechanisms, 1) a reduction in the release of acetylcholine in brain and peripheral muscarinic sites, 2) reversible inhibition of cholinesterase, and 3) blockade of central muscarinic receptors. All of these effects were achieved following administration of protective doses of clonidine. Furthermore, the muscarinic receptor down-regulation which occurs in response to elevated transmitter levels following soman administration is prevented in mice protected with clonidine [2]. This may simply be a reflection of clonidine's ability to limit acetylcholine release and postsynaptic receptor stimulation. It is not clear to what degree each of these three mechanisms contributes to the ability of clonidine to produce protection against the acute lethal actions of soman. However, several centrally acting α-adrenergic agonists of different chemical structures share this ability with clonidine, and each agonists' relative potency as a protective agent was related to its affinity for α-adrenergic binding sites labelled with [$^3$H]clonidine [9]. Also, the ability of clonidine to inhibit the biosynthesis of brain acetylcholine is mediated through α-adrenergic receptors [4]. It is this action of clonidine, therefore, which appears to predominate in its ability to protect against the acutely toxic actions of soman. It is possible that clonidine-induced protection of cholinesterase from irreversible inactivation by soman may provide a more chronic form of protection, that is, protection long after the clonidine is metabolized or excreted.

Along these lines, animals pretreated with clonidine that survive the soman challenge for several days appear behaviorally normal as compared with atropine-pretreated animals or saline-pretreated animals which survive an $LD_{50}$ dose of soman [12]. This apparent difference was observed even though protected animals may have received a higher dose of soman. Initially this finding might not seem noteworthy, since protected animals might be expected to have a better prognosis than nonprotected animals. However, soman is an irreversible inhibitor of AChE, and clonidine is a very short-acting drug, particularly in rodents [27]. In fact, animals protected to the same extent as clonidine with high doses (25 mg/kg) of atropine did not appear as behaviorally normal as the clonidine-pretreated animals. In rats [10], 0.5 mg/kg of clonidine produced a degree of protection equivalent to 6 mg/kg of atropine against lethality and soman-induced behavioral effects. The ability of a single dose of soman to induce behavioral abnormalities several days later has been reported [22]. In fact, the decrease in spontaneous motor activity induced by an $LD_{50}$ dose of soman in the rat was observed over 21 days. Such chronic toxic behavioral effects have also been observed following exposure to other organophosphate cholinesterase inhibitors in animals, and humans following accidental intoxication [for review, see, 28]. While the mechanism for this delayed toxicity is not clear, it has been reported that significant brain pathology can occur as early as 24 hrs following soman administration [14,38]. It has been suggested that the pathology may result from the severe convulsive activity present soon after soman administration [41]. Atropine pretreatment is only partially effective in reducing soman-induced convulsive activity and, hence, delayed brain pathology [39]. Clonidine pretreatment, however, was more effective than atropine in preventing the occurrence of soman-induced convulsive behavior, and survivors in the clonidine group were less behaviorally impaired than the atropine group [12].

High doses of atropine do not offer a substantial degree of protection against chronic toxicity. Of the three mechanisms of clonidine protection stated above, direct muscarinic receptor blockade is probably of minor importance. Since posttreatment with clonidine is not as effective as pretreatment (unpublished observation), the ability of clonidine to reduce acetylcholine release is an important contribution to its acute protective actions. The ability to protect ChE from irreversible inactivation [1,7] may be more important for protection against chronic soman toxicity.

Despite this effectiveness of clonidine in the rat, the maximal protective ratio (ratio of the $LD_{50}$ of protected/nonprotected animals) using clonidine alone is less than 2. Two classes of drugs commonly tested or employed as protective agents against ChE inhibitor toxicity are the muscarinic antagonists and carbamate ChE inhibitors. The latter group offers protection through its reversible inhibition of the enzyme which protects the active site from irreversible inactivation by soman [21,24]. Trihexyphenidyl was employed as the muscarinic antagonist in Part 1 of the present study, since this compound elicits greater central nervous system effects relative to peripheral effects than atropine [20]. Physostigmine is a centrally acting, short-acting carbamate cholinesterase inhibitor which can produce effective inhibition of brain enzyme [20]. Interestingly, clonidine reduces the side effects and toxicity associated with both muscarinic antagonists and physostigmine [9,37]. Therefore, when clonidine was employed, it was administered prior to the standard regimen. The purpose of **PART 1** of this study was to determine whether the addition of clonidine to a standard pretreatment protective regimen could offer added protection or benefit. In this study, the standard regimen employed was a mixture of physostigmine salicylate (150 μg/kg) and artane (trihexyphenidyl hydrochloride, 2 mg/kg).

As indicated above, both soman and clonidine can produce, respectively, toxicity and protection, through peripheral and central mechanisms. While clonidine is not particularly effective against selective peripherally acting ChE inhibitors, neither the extent of soman's central toxicity, nor the degree of clonidine's central protection have been directly investigated. In **PART 2** of this study, since we were primarily interested in studying the central toxicity of soman, we omitted the trihexyphenidyl from the regimen described in Part 1. Also, the centrally-acting physostigmine was replaced with the selective peripheral cholinesterase inhibitor pyridostigmine.

Finally in **PART 3**, we sought to confirm the ability of soman to produce central muscarinic receptor down-regulation (see above) in two additional species, rat and guinea pig. Changes in muscarinic receptor binding parameters in three brain regions were correlated with inhibition of brain cholinesterase (ChE) activity. Also, we examined the possibility that pretreatment with clonidine could inhibit the muscarinic receptor down-regulation produced by either acute, sub-acute, or chronic soman treatment.

## MATERIALS AND METHODS

### PART 1

Male Wistar rats weighing 270-300 g at the time of the experiment were obtained from Harland Sprague-Dawley, Indianapolis, IN and housed in an environmentally controlled room with free access to food (Wayne Rodent Bloks) and tap water, and were maintained on a 12-hr light-dark cycle. Animals were randomly assigned to one of four experimental groups: 1) normal controls receiving i.m. sterile saline injection followed 30 min later by s.c. saline injection; 2) rats receiving i.m. injection of saline followed 30 min later by one of several doses (60-110 μg/kg) of soman, s.c.; 3) rats receiving saline, i.m., followed 10 min later by the pretreatment regimen (physostigmine plus artane, see above), i.m., followed 30 min later by one of several doses of soman (160-300 μg/kg), s.c.; and 4) clonidine hydrochloride, 1 mg/kg, i.m., followed 10 min later by the pretreatment regimen, i.m., followed 30 min later by soman, s.c. Immediately after soman injection, rats were placed in open-top plastic cages for observational analysis as described previously [6,37]. Rats were observed for 30 sec during ten 3-min intervals. During each observation period, the appearance or expression of 12 behavioral signs were recorded on a checklist. These included the normal on-going behaviors: grooming, locomotor activity, rearing, and sniffing; as well as soman-promoted behaviors: abnormal body posture, chewing (vacuous), convulsions or jerks, hindlimb extension, muscle fasciculations, excessive salivation, Straub tail, and whole body tremor. The animals living for at least 24 hr after injection were considered survivors of the soman challenge. Two days after soman administration, rats were monitored in an open-field activity monitor (Digiscan) for 15 min, and six parameters of locomotor activity were recorded. Activity monitor measurements were repeated 1 week later. Three weeks after soman injection, rats were subjected to a passive-avoidance paradigm [17]. A standard shuttle cage with a guillotine door dividing the cage into a lighted and dark side was employed. A trial was initiated by raising the door and illuminating the 'safe' compartment. When the rat crossed over to the dark side, the door was lowered and an inescapable scrambled foot shock (1 mA for 5 sec) was delivered through the grid floor. The paradigm was repeated 24 hr later (no shock delivered), and the step-through latency was recorded. Rats remaining in the safe, lighted side for at least 5 min (cut-off) were considered to have learned the task.

Comparison between the means of several populations was performed using a one- or two-way ANOVA or an ANOVA for repeated measures, and the differences considered significant at the $p<0.05$ level. Student's t-test (two-tailed) was employed as a *post hoc* test and for the comparison of two groups of data. Multiple *Chi*-square analysis was used to compare the independence of checked behavioral signs.

Clonidine hydrochloride was purchased from Research Biologicals (Natick, MA). Physostigmine salicylate, artane (trihexyphenidyl hydrochloride), and soman (pinacolylmethylphosphonofluoridate) were supplied by the U.S. Army Medical Research and Development Command. Stock saline solutions (1 mg/ml) of soman were stored frozen at -70°C, and aliquots were diluted appropriately in sterile saline immediately prior to use. Soman solutions were maintained on ice during the experiment.

### PART 2

Male Wistar rats weighing 270-300 g at the time of the experiment were obtained from Harland Sprague-Dawley, Indianapolis, IN and housed in an environmentally controlled room with access to food (Wayne Rodent Bloks) and tap water, and maintained on a 12 hr light-dark cycle. Animals were randomly assigned to the various experimental groups: (S/S) sterile saline, i.m., (1ml/kg) followed 10 min later by saline, i.m., followed 20 min later by one of several doses of soman, s.c. The other regimens were, respectively: (S/P) saline - pyridostigmine, 0.13 mg/kg, i.m., - soman, s.c.; (C/P) clonidine, 1 mg/kg, i.m. - pyridostigmine - soman; (Gb/P) guanabenz, 5 mg/kg, i.m., - pyridostigmine - soman; (Gf/P) guanfacine,

5 mg/kg, i.m., -pyridostigmine- soman; (L/P) lofexidine, 1 mg/kg, i.m., - pyridostigmine - soman; (D/P) diazepam, 5 mg/kg, i.m., - pyridostigmine - soman. The timing of drug administration for each regimen was the same as that for the S/S group. Doses of clonidine and related analogs were determined from preliminary experiments in which doses of 0.5-5mg/kg were examined for maximum protective ability. The doses of pyridostigmine and diazepam were employed as previously determined to reduce soman-induced lethality and convulsive activity, respectively [33,43].

Immediately after soman injection, animals were placed in open-top plastic cages for observational analysis as described previously [37]. Animals were observed for 30 sec during ten 3-min intervals. During each observation period, the appearance or expression of 9 behavioral signs was recorded in a checklist. These included: abnormal body posture, convulsions or jerks, hindlimb extension, muscle fasciculations, excessive salivation, Straub tail, whole body tremor, teeth chattering, and chewing, (vacuous). In addition to these soman-promoted behaviors, four normal ongoing behaviors were noted, including grooming, sniffing, normal locomotor activity (exploratory behavior), and rearing. The animals living for at least 24 hr after injection were considered survivors of the soman challenge. Two days after soman administration, rats were monitored in an open-field activity monitor (Digiscan) for 15 min, and seven parameters of locomotor activity were recorded. [see 10,11,12]. Activity monitor measurements were repeated 1 week later.

Lethal dose-response (LD) curves were generated (3-4 doses per curve), and the $LD_{50}$ determined from the log dose plots by linear regression analysis. Comparison between the means of several populations was performed using a one- or two-way ANOVA or an ANOVA for repeated measures, and the differences were considered significant at the $P<0.05$ level. Student's *t*-test (two-tailed) was employed as a *post hoc* test and for the comparison of two groups of data. Multiple Chi-square analysis was used to compare the independence of checked behavioral signs.

Clonidine hydrochloride was purchased from Research Biologicals (Natick, MA). Guanfacine hydrochloride, guanabenz acetate, lofexidine hydrochloride and azepexole $Cl_2$ were gifts from, respectively, Sandoz Pharmaceuticals (East Hanover, NJ), Wyeth Laboratories (Philadelphia, PA), Merill Dow Pharmaceuticals (Cincinnati, OH) and Boehringer Ingleheim Ltd (Ridgefield, CT). Pyridostigmine (prydrochloride) and soman (pinacolylmethylphosphonofluoridate) were supplied by the U.S. Army Medical Research and Development Command. Stock saline solutions (1 mg/ml) of soman were stored frozen at -70°C and aliquots were diluted appropriately in sterile saline immediately prior to use. Soman solutions were maintained on ice during the experiment.

PART 3

Male Wistar rats weighing 270-300 g and male Hartly guinea pigs weighing 400 to 600 g at the time of the experiment were obtained from Harland Sprague-Dawley, Indianapolis, IN and housed in an environmentally controlled room with access to standard rat and guinea pig chow and tap water, and maintained on a 12 hr light-dark cycle. Animals were randomly assigned to the various experimental groups.

In acute studies, rats were injected s.c. with vehicle (sterile normal saline) or with 70 µg/kg of soman (a dose which is 0.85 of the 24 hr $LD_{50}$). Rats were decapitated at different time points after the injection, 30 min, 60 min, 120 min and 24 hr. Guinea pigs were injected with 35 µg/kg soman ($LD_{80-90}$) s.c. and decapitated 30 min later. For subacute injection of soman, a separate group of rats received six consecutive injections of soman (20 µg/kg, s.c., 0.24 of the $LD_{50}$) spaced 15 min apart. These rats were sacrificed 15 min after the last injection. For chronic studies, soman (20, 30, and 40 µg/kg) doses representing respectively 0.24, 0.36, and 0.48 of the 24 hr $LD_{50}$, s.c. once daily for 7 days. On the 7th day, rats were decapitated 15 min after the last injection. In each study group, brains were removed and dissected into the cortex, midbrain, and hindbrain (pons and medulla). Tissue was weighed and frozen at -70°C prior to assay.

Muscarinic receptor binding was measured using [N-methyl-$^3$H]scopolamine methyl chloride ([$^3$H]MS) and an assay medium containing 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 75 µg membrane protein in a total volume of 2 ml. Incubations were carried out for 90 min at room temperature. Non-specific binding was determined in the presence of 10 µM atropine. Tissue consisted of rat or guinea pig cortex, midbrain, or hindbrain homogenized in Tris-Mg buffer and spun at 20,000 g for 20 min. The pellet was resuspended in fresh buffer and used without further treatment. To measure the Bmax and Kd of muscarinic acetylcholine receptors (mAChRs) in the brain, (MS)(10 nM-0.01 nM) was incubated with 0.10 mg protein at room temperature for 90 min, using 10 µM atropine to obtain the nonspecific binding. In these experiments, receptor density was determined by measuring specific [$^3$H]MS binding at three concentrations (0.1, 0.32, and 1 nM) and calculating the total number of binding sites assuming the [$^3$H]MS dissociation constants determined from earlier saturation experiments in control animals. This method was applicable since preliminary experiments ascertained that the soman and the other drugs do not alter muscarinic receptor binding affinity [2]. Binding data were fitted to a one-site model [B=Bmax*C/(C+Kd), where B is the bound fraction of label and C is the ligand concentration] using a nonlinear curve fitting program (Tablecurve, Jandel Scientific, San Rafael, CA). Protein concentration was measured using the Bio-Rad Protein Assay (Richmond, CA) system, using bovine albumin as standard. Statistical analysis of the resulting Bmax and Kd was performed using a two-tailed Student's t test; and *statistical significance was achieved when* $p<0.05$.

ChE activity was determined for each brain tissue sample. Phosphate buffer, pH 7.0 (10 ml/g, wet weight) was added and the tissue homogenized. ChE activities were determined spectrophotometrically in whole tissue homogenate by the method of Ellman [16]. The homogenized brain aliquot was introduced into a cuvette containing the reaction mixture: 50 mM Tris-HCl, pH 8, containing 11 mM acetylthiocholine iodide (substrate), and 6.9 mM Dithiosnitrobenzoic acid. The absorbance at 412 nm was recorded for 2 min.

## RESULTS

### PART 1

Administration of soman to saline-pretreated rats resulted in dose-dependent increase in lethality over a very narrow concentration range (Fig. 1). The $LD_{50}$ for this control group was 80 µg/kg. Pretreatment with the standard protective regimen (physostigmine plus artane) increased the $LD_{50}$ threefold, i.e., resulted in a protective ratio of 3. When clonidine administration preceded the standard regimen, there was no significant change in the protective ratio. However, in two preliminary experiments in which 220 and 240 µg/kg ($LD_{50}$) of soman were employed, clonidine was administered after the standard regimen but 10 min prior to soman. In this case all animals survived the soman challenge. These results indicate that time of administration may be important in maximizing clonidine's acute protective effects. However, clonidine was the first agent administered in the remainder of the experiments described below.

None of the soman-promoted behaviors were observed in normal controls (Table 1). For clarity of presentation, only two observational epochs are presented in Table 1: 0-3 min, representing the time of maximal expression of normal activity, and 15-17 min, representing the time of maximal expression of soman-promoted behaviors. The behavior of saline-pretreated, soman animals was similar to controls during the first 3 min after injection. This probably reflects the time required for adequate drug absorption and distribution. During the 15 to 17 - min time period, however, normal behaviors, such as grooming and sniffing were absent, and all soman-promoted behaviors were expressed, except for muscle fasciculations. Pretreatment with the standard regimen did not reverse soman-induced inhibition of normal behaviors but did inhibit the expression of several soman-promoted behaviors. The incidences of abnormal posture and tremor were not reduced, and the incidence of muscle fasciculations actually increased. When clonidine was included with the standard pretreatment regimen, no additional benefit (or worsening) was observed (data not shown), except for a significant reduction in soman-induced tremor, as is illustrated in Figure 2.

When surviving rats were examined for performance in an open field 48 hr after soman administration, those rats receiving soman doses lower than the $LD_{50}$ exhibited behavior not significantly different from controls. In contrast, rats surviving the $LD_{50}$ dose exhibited significantly depressed activity. The data for horizontal activity are presented as an example in Figure 3. This profile was characteristic of all motor parameters measured (data for the other parameters not shown). Since there were too few survivors in the $LD_{50}$ or greater group to make a meaningful statistical comparison with the clonidine plus standard regimen group, and since there was no significant difference among the activities observed for this group, values for these three highest doses were combined (Table 2). Animals from the standard regimen treated with soman displayed significantly reduced locomotor activity in most parameters measured. Addition of clonidine to the standard regimen did not completely reverse the effects of soman, but these animals exhibited a significantly improved motor activity score in three of the parameters: horizontal activity, total distance traveled, and movement time.

Surviving rats were then examined for performance in the open field activity monitor 9 days after soman administration. In this case animals receiving the standard regimen exhibited normal motor activity in all parameters and across all doses of soman. Horizontal activity is presented as an example in Figure 4. Addition of clonidine to the standard regimen resulted in a similar profile of activity (data not shown).

In the last experimental series, the behavior of 1) saline control animals, 2) saline-pretreated animals receiving an $LD_{50}$ (80 µg/kg) dose of soman, 3) saline-pretreated animals receiving the standard protective regimen and an $LD_{50}$ (240 µg/kg, in protected animals) dose of soman, and 4) clonidine-pretreated animals receiving the protective regimen and 240 µg/kg of soman were examined in a standard passive-avoidance paradigm 3 weeks after soman administration (Fig 5). Training latencies were not different among the groups (Fig 5). In contrast, animals receiving saline plus the 80 µg/kg dose of soman exhibited reduced step-through latencies on the test day, suggesting memory impairment. In rats receiving the standard regimen and the higher dose of soman, latencies were improved with respect to non-protected

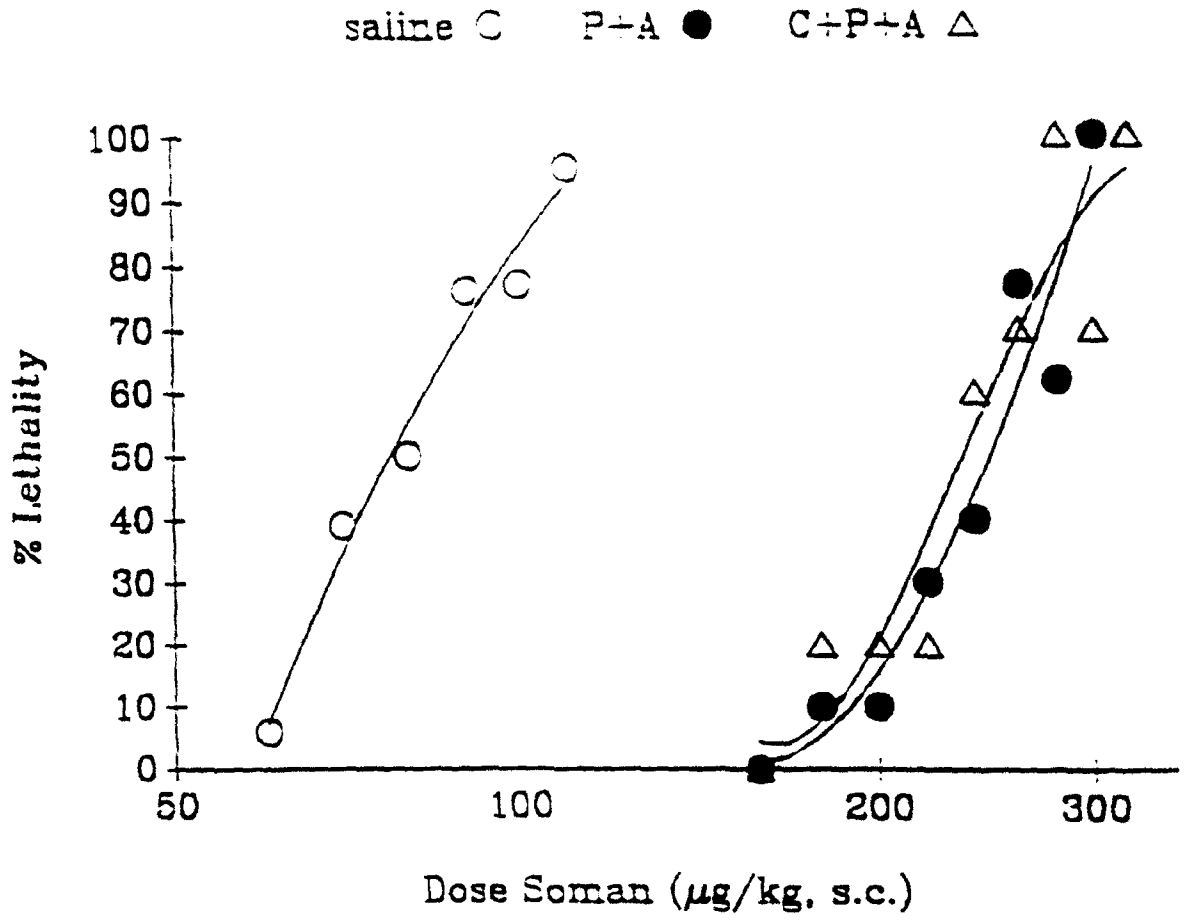


Figure 1

Lethal dose-response (LD) relationship to soman in rats pretreated with saline, a standard pretreatment regimen (P+A), and clonidine before the standard pretreatment regimen (C+P+A). The $LD_{50}$ for the unprotected group was 80 μg/kg which increased threefold with the standard pretreatment regimen. Preceding the standard pretreatment regimen with clonidine did not alter the LD curve. Each point was derived from 10-14 animals.

TABLE 1

EFFECT OF A STANDARD PROTECTIVE REGIMEN AGAINST THE SOMAN-INDUCED ACUTE BEHAVIORAL TOXICITY

The percentage of animals in each group displaying the indicated behavior is listed.

| | REGIMEN[a]/TIME INTERVAL (min) | | | | | |
|---|---|---|---|---|---|---|
| | SAL-SAL | | SAL+SOM(80)[b] | | SAL+P+A+SOM(240)[c] | |
| BEHAVIOR | 0-3 | 15-17 | 0-3 | 15-17 | 0-3 | 15-17 |
| GROOMING | 7 | 29 | 0 | 0 | 0 | 0* |
| LOCOMOTOR ACTIVITY | 86 | 57 | 100 | 0* | 100 | 0* |
| REARING | 86 | 50 | 100 | 0* | 100 | 0* |
| SNIFFING | 93 | 86 | 100 | 0* | 100 | 0* |
| ABNORMAL POSTURE | 0 | 0 | 0 | 100 | 0 | 88 |
| CHEWING | 0 | 0 | 0 | 23 | 0 | 0 |
| CONVULSIONS/JERKS | 0 | 0 | 0 | 46* | 0 | 0 |
| HINDLIMB EXTENSION | 0 | 0 | 0 | 77* | 0 | 0 |
| MUSCLE FASCICULATIONS | 0 | 0 | 0 | 8 | 0 | 88* |
| EXCESSIVE SALIVATION | 0 | 0 | 0 | 100* | 0 | 0 |
| STRAUB TAIL | 0 | 0 | 0 | 100* | 0 | 0 |
| TREMOR | 0 | 0 | 0 | 100* | 0 | 63* |

a SAL = saline; P = physostigmine salicylate, 150μg/kg, i.m., 30 min prior to soman; A = artane (trihexyphenidyl hydrochloride), 2 mg/kg, im., 30 min prior to soman; SOM = soman, 240-300 μg/kg, s.c.

b Saline-pretreated rats received an $LD_{50}$ dose (80 ug/kg) of soman.

c Physostigmine- and artane-pretreated rats received an $LD_{50}$ dose (240μ/kg) of soman. n = 14 for SAL-SAL; n = 13 for SAL+SOM(80) group and n = 11 and 8 for the 0-3 and 15-17 min. intervals, respectively, for the SAL+P+A+SOM(240) group.

* Significantly different from SAL-SAL group, $p<0.05$.

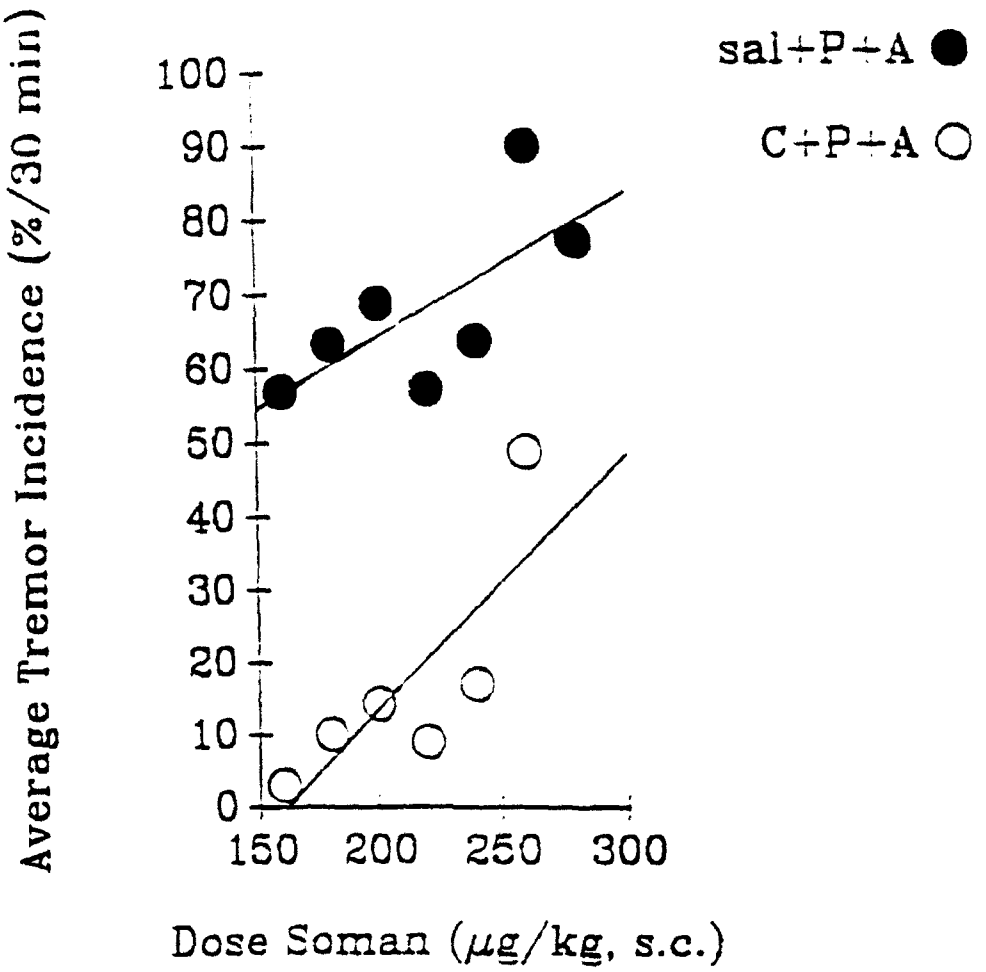


Figure 2

Effect of clonidine (C+P+A) added to the standard pretreatment regimen (sal+P+A) on soman-evoked tremor activity. For ease of presentation, incidences recorded at each observation period were averaged over the 10 periods. Addition of clonidine to the regimen resulted in a significant reduction in soman-induced tremor. Based upon ten animals per dose.

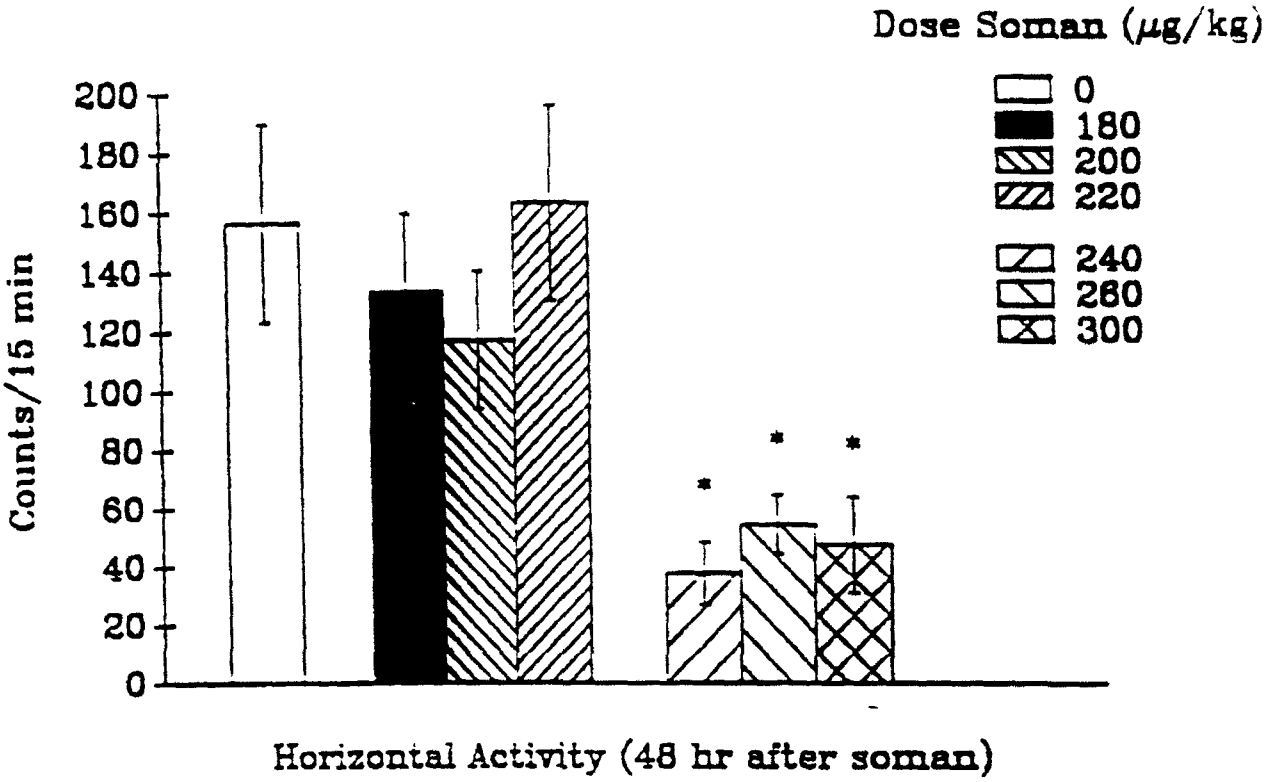


Figure 3

Effect of pretreatment with the standard regimen on open-field activity of control and soman-treated rats. Animals were monitored 48 hr after soman administration; horizontal activity is presented as an example. * = significantly different from control values (0 dose), $p<0.05$. Vertical lines indicate the S.E.M. N = 14, 9, 9, 7, 5, 3, and 4, respectively, for the control and six doses of soman.

TABLE 2

EFFECT OF ADDITION OF CLONIDINE TO A PROTECTIVE REGIMEN AGAINST SOMAN-INDUCED CHRONIC (48 HR) BEHAVIORAL TOXICITY

| | Pretreatment-Treatment Regimens[a] | | |
|---|---|---|---|
| ACTIVITY | SAL - SAL | SAL+P+A+SOM | C+P+A+SOM |
| Horizontal | 156.3 ± 33.3 | 44.9 ± 7.1[c] | 79.3 ± 20.6[c,d] |
| Vertical | 14.3 ± 4.1 | 4.4 ± 0.67[c] | 5.5 ± 1.6[c] |
| Total Distance | 54.3 ± 13.5 | 13.4 ± 2.2[c] | 24.5 ± 6.2[c,d] |
| Movement Time | 20.1 ± 4.0 | 7.7 ± 1.2[c] | 12.0 ± 2.7[d] |
| Stereotype Time | 6.2 ± 1.6 | 3.7 ± 0.54 | 4.2 ± 0.74 |
| Time in Center | 1.6 ± 0.65 | 1.8 ± 0.37 | 1.1 ± 0.33 |
| n | 7 | 12 | 7 |

[a] SAL = saline; P = physostigmine salicylate, 150 μg/kg, i.m., 30 min prior to soman; A = artane (trihexyphenidyl hydrochloride), 2 mg/kg, i.m., 30 min prior to soman; C = clonidine hydrochloride, 1 mg/kg, 40 min prior to soman; SOM - soman, 240-300 μg/kg, s.c.

[b] Horizontal and Vertical activity units = counts/min; Total Distance travelled units = inches; Movement time, Stereotype time and Time in Center units = sec.

[c] Significantly different from SAL-SAL group.

[d] Significantly different from SAL+P+A+SOM group. Each value refers to the mean ± S.E.M.

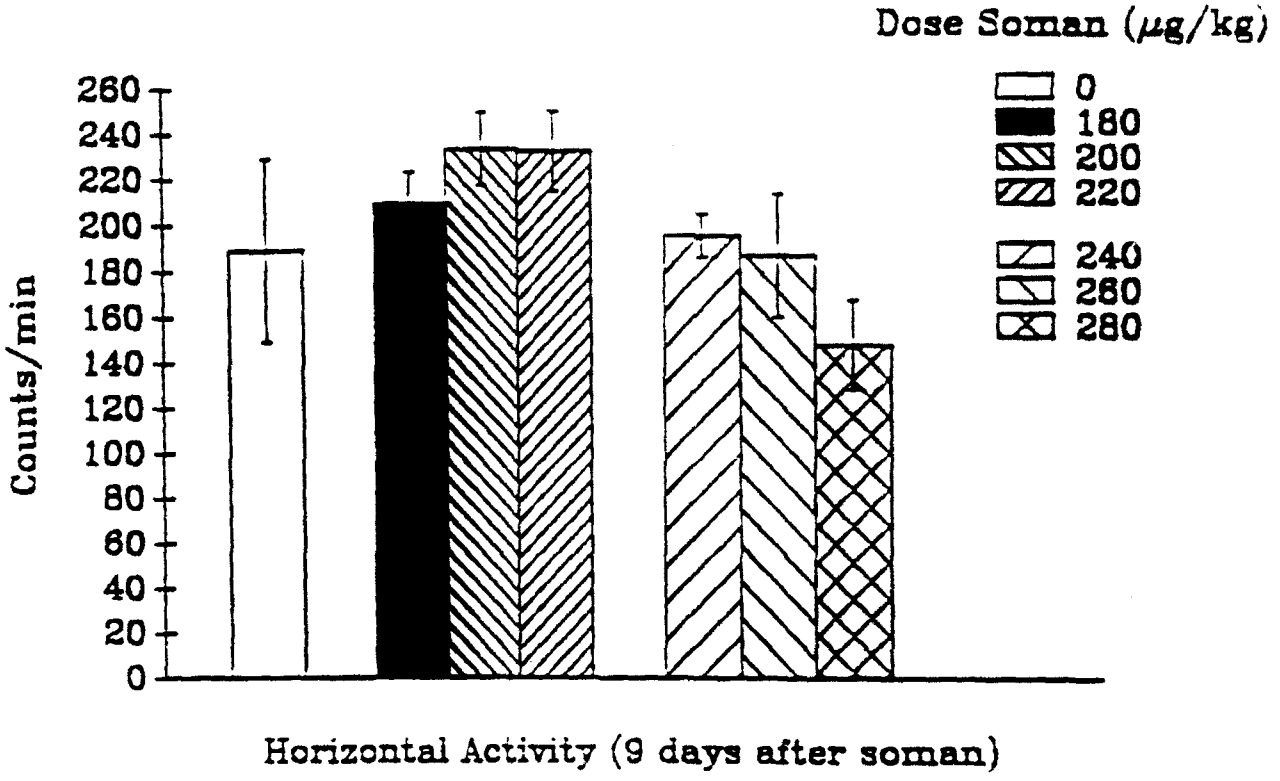


Figure 4

Effect of pretreatment with the standard regimen on open-field activity of control rats and soman-treated rats. Animals were monitored 9 days after soman administration. In this case, animals receiving the standard pretreatment regimen exhibited normal motor activity in all parameters (horizontal activity is presented as an example) and across all doses of soman. N = 14, 9, 9, 6, 5, 3, and 4, respectively, for the control and six doses of soman.

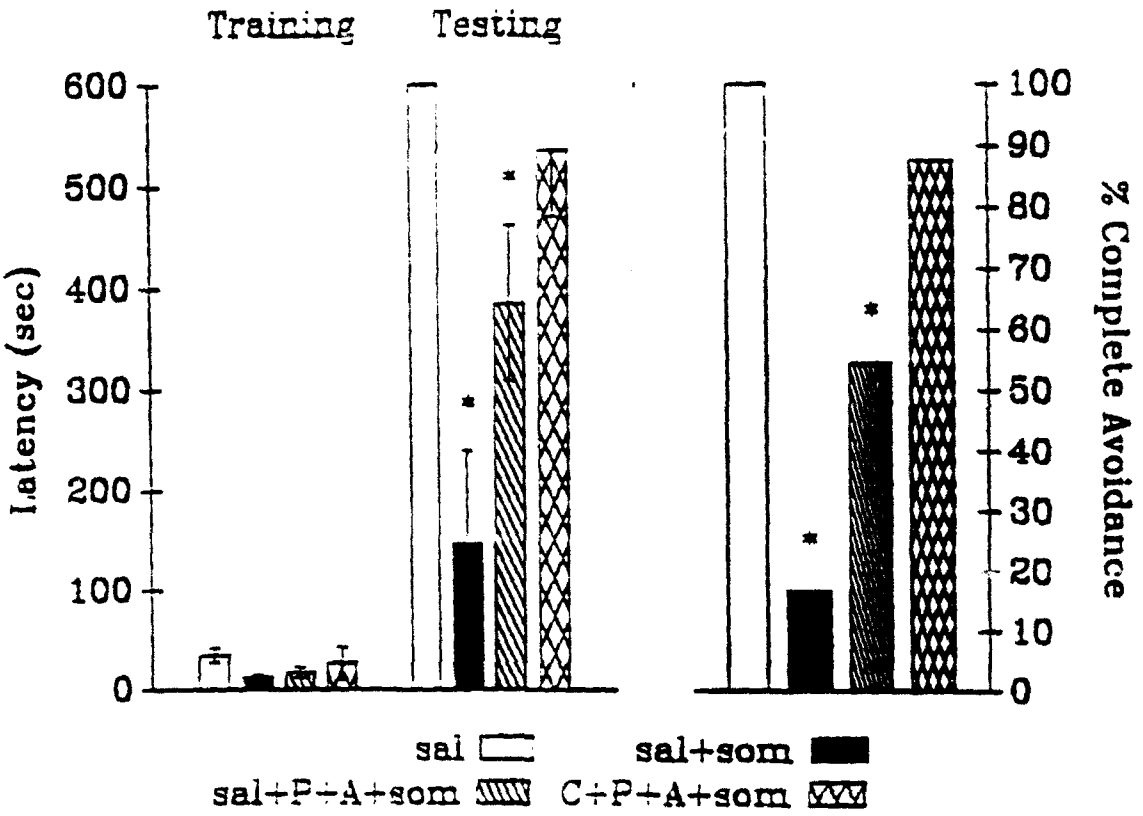


Figure 5

Behavior of saline control animals (sal), N = 8; saline-pretreated animals receiving an $LD_{50}$ (80 µg/kg, N = 6) dose of soman (sal + som); saline-pretreated animals receiving the standard protective regimen and an $LD_{50}$ (240 µg/kg, N = 11) dose of soman (sal+P+A+som); and clonidine-pretreated animals receiving the protective regimen and 240 µg/kg, N = 8 of soman (C+P+A+som); in a standard passive-avoidance paradigm 3 weeks after soman administraiton. * = significantly different from control (sal) group, $p<0.05$.

rats: however, they were still significantly reduced compared with control (saline only) values. Including clonidine in the pretreatment regimen resulted in test latencies which were not significantly different from control values. The same profile is exhibited when the data are expressed in terms of percentage of animals displaying complete avoidance (i.e., remaining in the bright compartment for at least 5 min).

PART 2

In control, nonprotected rats, soman produced a dose-related increase in lethality over a narrow dose range 60-280 μg/kg. Because of slight differences in animal sensitivity to soman over the course of the study, three LD curves were generated at different times for comparison with potential protective agents. The $LD_{50}$ for soman in rats averaged 108.3±19.9 μg/kg. The Protective Ratio (PR) ($LD_{50}$ obtained in protected animals/$LD_{50}$ obtained in nonprotected animals) for each of the combinations are listed in Table 3. Despite the fact that in our earlier study [8] and in the preliminary experiments of this study all analogs offered significant protection against soman lethality when employed as the sole protectant, only clonidine, guanfacine, and guanabenz offered significant protection in addition to that provided by pyridostigmine alone. Note that diazepam was included in this portion of the study to provide contrast with the central effects of clonidine. Diazepam has previously been demonstrated to inhibit the convulsive or seizure activity produced by soman, but does not usually provide significant protection against the acute lethal effects.

In protected, and to various degrees in nonprotected rats, soman produced significant signs and symptoms of cholinesterase inhibitor poisoning. Data presented in Table 4 represent the maximal expression of behaviors and symptoms elicited by soman within the 30 min observation period after injection. Data were compared using doses of soman in each regimen resulting in approximately similar rates of survival (40-60%). Values for each parameter measured in unprotected soman-injected rats are presented for comparison, but since the $LD_{50}$ doses for unprotected animals were much lower than for protected animals, these values are not employed for statistical comparison. The hypothesis to be tested was whether the addition of an $\alpha_2$-adrenergic agonist provided a greater degree of protection than that using pyridostigmine alone. Therefore, statistical comparisons were made with the object of testing this hypothesis. In general, addition of each agonist to the regimen resulted in some benefit compared with pyridostigmine alone. The beneficial effects of lofexidine and azepexole were surprising in this regard, since they did not enhance the PR. Nevertheless, the addition of an $\alpha_2$ agonist generally resulted in a reduced incidence of convulsions/jerks, and hindlimb extension, overt signs of seizure activity in rats. Other symptoms controlled by the addition of $\alpha_2$ agonists included excessive salivation and tremor (except for guanabenz which was not effective in this case). It should also be pointed out that for symptoms in which clonidine or an analog significantly reduced the prevalence of a soman-induced behavior, the average time after soman injection for maximal expression of the symptom was often greatly prolonged. For example, clonidine doubled the time for maximal expression of whole body tremor in the group as compared with both nonprotected animals and as compared with pyridostigmine pretreated rats. Like clonidine, diazepam was effective in reducing the prevalence of convulsive behavior, abnormal posture, and Straub tail; however, it did not increase the numbers of survivors following soman administration (Table 3) and was not effective in reducing the prevalence of hindlimb extension or excessive salivation.

Normal behaviors which were inhibited by soman included grooming, sniffing, locomotor activity (general exploratory behavior), and rearing. Our past experience has demonstrated that it is much more difficult to reverse soman's inhibition of normal on-going behavior than it is to reverse soman-evoked abnormal behavior with pretreatment regimens. This was also the case in this study; however, only the regimen which included clonidine resulted in a significant reversal of soman-induced inhibition of normal sniffing and locomotor activity (Fig. 6). None of the other pretreatment combinations offered significant protection in this regard.

To determine whether addition of agonist could provide any additional benefit in terms of long-term toxicity in survivors, we examined rats in an automated locomotor activity chamber (Digiscan) at 2

TABLE 3

THE RELATIVE PROTECTIVE ABILITY OF CLONIDINE AND ITS ANALOG TO SOMAN POISONING IN RATS

| REGIMEN | PROTECTIVE RATIO |
|---|---|
| SALINE + PYRIDOSTIGMINE + SOMAN | 1.44 |
| CLONIDINE + PYRIDOSTIGMINE + SOMAN | 2.00 |
| GUANFACINE + PYRIDOSTIGMINE + SOMAN | 1.80 |
| GUANABENZ + PYRIDOSTIGMINE + SOMAN | 2.21 |
| LOFEXIDINE + PYRIDOSTIGMINE + SOMAN | 1.21 |
| AZEPEXOLE + PYRIDOSTIGMINE + SOMAN | 1.44 |
| DIAZEPAM + PYRIDOSTIGMINE + SOMAN | 1.59 |

TABLE 4

PREVALENCE OF SOMAN-EVOKED BEHAVIORS AT THE TIME OF MAXIMAL EXPRESSION.

| | Pretreatment Regimen | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | S/S | S/P | C/P | Gb/P | Gf/P | L/P | A/P | D/P |
| Abnormal Posture | 93(10) | 93(7) | 40(21)* | 100(6) | 100(8) | 100(6) | 100(3) | 0* |
| Convulsions/ Jerks | 85(10) | 100(8) | 10(15)* | 60(21)* | 50(23)* | 44(24)* | 50(18)* | 10(6)* |
| Hindlimb Extension | 13(6) | 55(9) | 10(3)* | 30(12) | 13(8)* | 0* | 10(6)* | 37(18) |
| Muscle Fasciculations | 3(3) | 6(2) | 20(3) | 50(6)* | 13(8) | 0 | 0 | 87(18) |
| Excessive Salivation | 100(10) | 100(9) | 20(15)* | 90(24) | 60(23)* | 44(24)* | 50(15)* | 100(15) |
| Straub Tail | 67(7) | 50(11) | 0* | 60(21) | 33(21) | 22(24) | 30(12) | 0* |
| Tremor | 100(6) | 100(7) | 20(12)* | 100(27) | 47(20)* | 78(24)* | 80(24)* | 100(3) |
| Teeth Chattering | 7(9) | 13(6) | 0 | 0 | 0 | 0 | 0 | 0 |
| Chewing | 43(8) | 46(11) | 20(12) | 20(18) | 16(12)* | 0* | 50(9) | 80(3) |
| N | 30 | 35 | 10 | 10 | 20 | 10 | 10 | |

S/S = saline, i.m., followed 10 min later by saline, i.m., followed 20 min later by soman, s.c. at a dose producing lethality within 24 hr in 40-60% of the animals (LD 40-60). The other regimens were respectively: S/P = saline - pyridostigmine, 0.13 mg/kg - soman; C/P = clonidine, 1 mg/kg - pyridostigmine - soman; Gb/P = guanabenz, 5 mg/kg - pyridostigmine - soman; Gf/P = guanfacine, 5 mg/kg - pyridostigmine - soman; L/P = lofexidine, 1 mg/kg - pyridostigmine - soman; A/P = azepexole, 1 mg/kg - pyridostigmine - soman; D/P = diazepam, 5 mg/kg - pyridostigmine - soman.

Each value represents the maximal frequency of expression of symptoms over the 30 min observation period. The numbers in parenthesis indicate the average time (min) after soman injection for maximal expression of the symptom.

* = significantly different from S/P group, $p<0.05$.

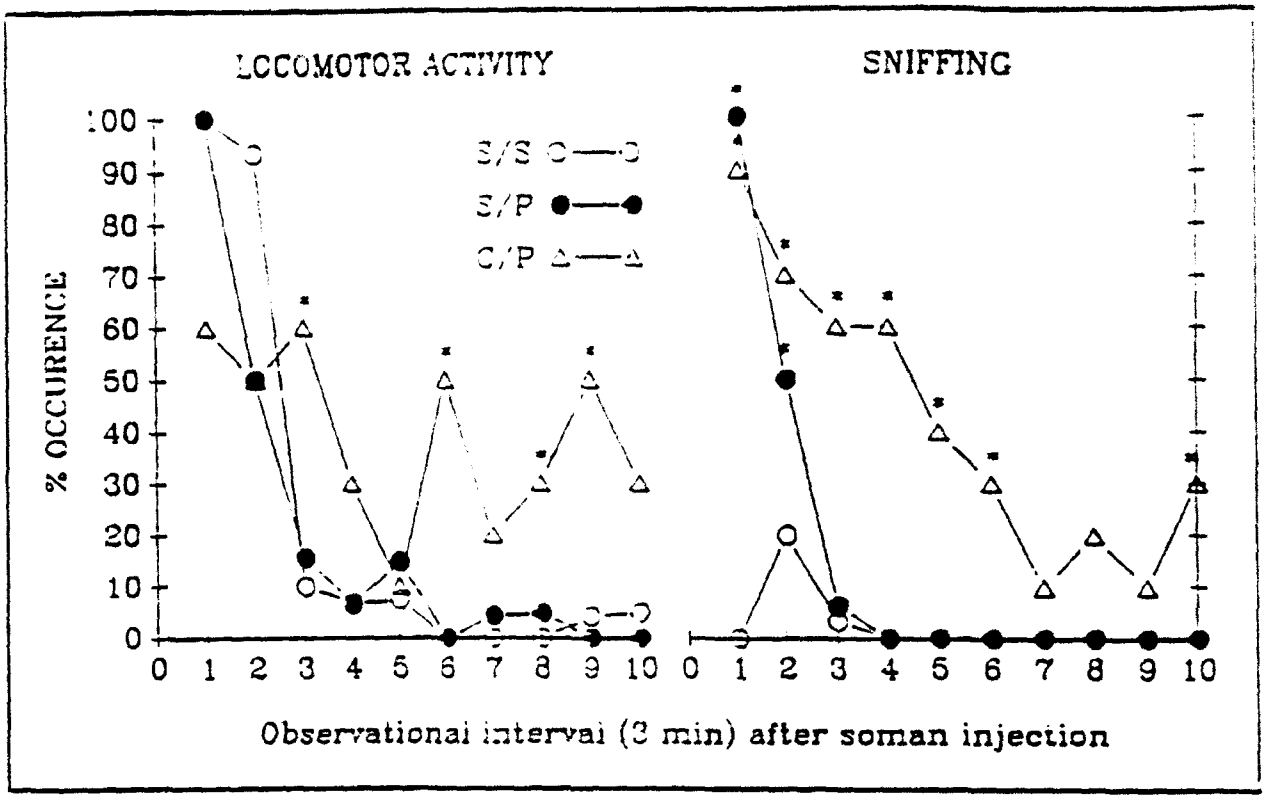


Figure 6

Effect of clonidine added to a pyridostigmine pretreatment regimen on soman-evoked inhibition of normal motor activity in an open field. S/S - sterile saline, i.m. (1 ml/kg), followed 10 min later by saline, i.m., followed 20 min later by soman, ($LD_{10-60}$) s.c., S/P = saline - pyridostigmine, 0.13 mg/kg, - i.m. - soman, s.c.; C/P = clonidine, 1 mg/kg, i.m. - pyridostigmine - soman.
* = significantly different ($P<0.05$) from S/S and S/P groups.

TABLE 5

OPEN-FIELD MOTOR ACTIVITY 2 DAYS AFTER SOMAN INJECTION

| | Pretreatment Regimen | | | | | | |
|---|---|---|---|---|---|---|---|
| | S/S | S/P | C/P | Gb/P | Gf/P | L/P | D/P |
| HOR (counts) | 399.8 ±66.3 | 1139.4 ±257.5 | 1572.3 ±484.2 | 1306.7 ±722.7 | 1710.0 ±223.3 | 924.3 ±446.8 | 1465.8 ±270.7 |
| VER (counts) | 3.01 ±1.34 | 15.6 ±10.7 | 116.2* ±56.9 | 2.40 ±0.81 | 132.0* ±29.7 | 59.0 ±41.5 | 146.5* ±39.8 |
| TD (inches) | 376.4 ±72.6 | 1019.3 ±196.6 | 1533.7 ±480.8 | 1256.0 ±673.9 | 1530.0* ±213.7 | 660.5 ±381.1 | 1391.2 ±266.8 |
| MT (sec) | 78.6 ±13.9 | 132.2 ±20.1 | 251.8 ±106.7 | 120.6 ±45.8 | 218.4* ±21.3 | 105.3 ±44.4 | 162.7 ±27.2 |
| RT (sec) | 346.8 ±16.0 | 265.9 ±20.2 | 239.0 ±47.2 | 290.0 ±43.2 | 197.1* ±21.5 | 309.8 ±46.5 | 241.9 ±30.2 |
| STER (sec) | 18.9 ±3.6 | 29.0 ±5.9 | 50.7* ±10.1 | 32.6 ±17.8 | 64.8* ±9.5 | 24.3 ±13.1 | 42.8 *6.9 |
| TIC (sec) | 100.6 ±35.5 | 37.6 ±15.6 | 39.7 ±8.7 | 61.8 ±19.2 | 3.4 ±1.3 | 10.0* ±4.9 | 83.1 ±38.1 |
| N | 13 | 16 | 6 | 5 | 9 | 4 | 17 |

S/S = saline, i.m., followed 10 min later by saline, i.m., followed 20 min later by soman, s.c. (LD 40-60). The other regimens were respectively: S/P = saline-pyridostigmine, 0.13 mg/kg - soman; C/P = clonidine, 1 mg/kg pyridostigmine - soman; Gb/P = guanabenz, 5 mg/kg - pyridostigmine - soman; Gf/P = guanfacine, 5 mg/kg - pyridostigmine - soman; L/P = lofexidine, 1 mg/kg - pyridostigmine - soman; D/P = diazepam, 5 mg/kg pyridostigmine - soman.

HOR = horizontal activity; VER = vertical activity; TD = total distance traveled; MT = movement time; RT = rest time; STER = stereotype time; TIC = time in center.

*Each value indicates the mean* ± S.E.M.

* = significantly different from S/P group, $p<0.05$.

TABLE 6

OPEN-FIELD MOTOR ACTIVITY 9 DAYS AFTER SOMAN INJECTION

| | Pretreatment Regimen | | | | | | |
|---|---|---|---|---|---|---|---|
| | S/S | S/P | C/P | Gb/P | Gf/P | L/P | D/P |
| HOR (counts) | 1304.8 ±313.8 | 1041.0 ±313.9 | 2782.0* ±645.2 | 4528.5 | 1947.0* ±245.6 | 1755.3 ±275.2 | 1690.3 ±252.1 |
| VER (counts) | 31.8 ±10.2 | 122.3 ±3.23 | 362.5* ±37.0 | 179.5 ±2.5 | 236.1* ±30.0 | 125.0 ±39.7 | 150.3 ±32.9 |
| TD inches | 1528.4 ±401.8 | 3100.6 ±395.0 | 2595.3 ±651.0 | 4307.0 ±316.0 | 1953.8# ±298.3 | 1680.5# ±292.2 | 1415.9# ±158.7 |
| MT (sec) | 232.8 ±61.4 | 313.6 ±8.4 | 260.0# ±17.5 | 347.0 ±41.0 | 292.7 ±44.4 | 234.0# ±26.7 | 211.1# ±19.3 |
| RT (sec) | 221.8 ±38.9 | 110.1 ±9.1 | 153.8# ±16.4 | 80.5 ±48.5 | 182.3# ±28.0 | 186.3# ±26.6 | 209.4# ±19.3 |
| STER (sec) | 46.0 ±13.0 | 89.8 ±6.8 | 82.0 ±7.7 | 117.5 ±7.5 | 79.7 ±7.3 | 63.5 ±11.7 | 60.2# ±8.0 |
| TIC (sec) | 65.1 ±49.1 | 27.5 ±6.2 | 10.8* ±3.8 | 7.5 ±0.5 | 3.2* ±1.1 | 3.5* ±2.4 | 8.9* ±2.8 |
| N | 8 | 11 | 4 | 2(n.s.) | 9 | 4 | 15 |

See legend for Table 3.
Each value indicates the mean ± S.E.M. (n.s.) = not sufficient for statistical analysis.
* = significant improvement in performance with respect to S/P group, $p<0.05$.
# = significant decrement in performance with respect to S/P group, $p<0.05$.

TABLE 7

CHANGE IN BODY WEIGHT OVER 3 WEEKS FOLLOWING SOMAN INJECTION

| | Starting Weight (g) | Change in Weight (g) | | |
|---|---|---|---|---|
| | Day 0 | Day 2 | Day 9 | Day 21 |
| S/S | 272 ± 2 | -60 ± 2 (40) | -46 ± 15 (27) | 41 ± 9 (23) |
| S/P | 259 ± 3 | -55 ± 1 (46) | -11 ± 10 (31) | 73 ± 7 (31) |
| C/P | 265 ± 3 | -39 ± 12 (60) | 21 ± 24 (40) | 102 ± 12 (40) |
| Gb/P | 268 ± 3 | -95 ± 10* (50) | -65 (20) | 39 (20) |
| Gf/P | 268 ± 3 | -21 ± 7* (50) | 32 ± 6* (45) | 86 ± 5 (45) |
| L/P | 268 ± 3 | -39 ± 16 (40) | 7 ± 23 (40) | 105 ± 18 (40) |
| A/P | 270 ± 5 | -8 ± 15* (50) | ND | ND |

See legend for Table 3.
Each value indicates the mean ± S.E.M. Numbers in parentheses indicate the percent survival.
* = significantly different from S/P group, $p<0.05$.
ND = not determined.

and 9 days after soman injection. Rats surviving $LD_{40-60}$ doses of soman were employed for comparison. The results for 2 days after soman are presented in Table 5 (for technical reasons, chronic tests of azepexole-treated rats are not presented). The use of pyridostigmine appeared to allow for a greater recovery of locomotor activity (activity scores consistent with increased motor activity) compared with soman alone (even though the dose of soman in unprotected rats was much lower). Addition of an $\alpha_2$-adrenergic agonist to the regimen resulted in an even greater recovery of locomotor activity for guanfacine and clonidine. For clonidine, this improvement was reflected primarily in the significant enhancement of vertical activity and normal stereotyped activity. Diazepam's beneficial actions were limited only to a significant improvement in vertical activity. Interestingly, guanabenz and lofexidine did not offer any additional improvement compared with pyridostigmine alone.

At 9 days after soman, in all groups, the various components of motor activity were improved in survivors compared with the 2 day measures (Table 6). Also, at 9 days there was no consistent improvement in performance observed in animals receiving a regimen containing clonidine or an analog with respect to pyridostigmine alone. However, it should be noted that higher doses of soman were employed in animals protected with analogs (except for lofexidine). In contrast, addition of diazepam with pyridostigmine treatment was associated with significantly reduced performance in four of the seven measures compared with pyridostigmine alone (even though the doses of soman employed were lower for diazepam than for the $\alpha_2$ agonists.

Rats lost a very consistent amount of weight at 2 days after soman injection (Table 7). Pyridostigmine pretreatment alone did not reverse this loss; however, rats receiving clonidine, guanfacine, and azepexole as part of the protective regimen lost significantly less weight than unprotected animals. Although the results were more variable on the 9th day after soman, there was a trend for clonidine-treated rats to gain more weight, and guanfacine rats significantly gained more weight than the pyridostigmine (alone) animals. There was no difference in body weight among any of the protected groups by the 21st day after soman injection.

PART 3

In rats, the injection of soman (70 μg/kg, s.c.) resulted in a 90% inhibition of the ChE activities in all the brain areas tested (Fig. 7). The density (Bmax) for mAChRs following a single injection of soman was significantly reduced at 2 hr after injection ($p<0.05$) in the cortex and hindbrain. Bmax values, however, returned to baseline within 24 hr (Table 8). Interestingly, sub-acute treatment with a sub-lethal dose of soman in rats (see the Method section) also decreased the density of muscarinic receptors. Although the soman administration paradigms were different, both groups that exhibited decreased Bmax values were sacrificed approximately 2 hr after the first injection. In both cases the density of muscarinic receptors was reduced by about 15% for the cortex and 17% for the hindbrain (the midbrain was also reduced by 18% for sub-acute injections). In guinea pigs, soman (35 μg/kg, s.c., $LD_{50-90}$) resulted in a 90% inhibition of the ChE activity in three brain areas (Table 9). This treatment did not change the mAChR density and the affinity in the areas measured (Table 9).

Chronic administration of soman (20-40 μg/kg, s.c.) produced a dose-related inhibition of ChE activity with the highest dose almost abolishing the ChE activity in the rat cortex (Table 10). But this long-term treatment procedure did not down-regulate mAChRs. Chronic treatment of rats with any of the three doses of soman did not alter the [$^3$H]MS binding density in the areas measured (Table 10). Similar results were obtained with midbrain and hindbrain regions as observed for the cortex. Also, no changes in binding affinity were observed in any of the brain regions to any of the treatment regimens.

In the final series of experiments, we sought to confirm the protective action of clonidine in soman-induced muscarinic receptor down-regulation as had been observed earlier in mice [2]. Rats were administered saline (vehicle) or clonidine (1 mg/kg, s.c.) 5 min prior to 70 μg/kg of soman, s.c. The animals were sacrificed 2 hr after soman and the brain regions obtained and subjected to analysis for muscarinic receptor density as described above. As indicated in Figure 8, clonidine pretreatment reversed

the soman-induced muscarinic receptor down-regulation in cortex and hindbrain. Unexpectedly, clonidine enhanced binding in the midbrain.

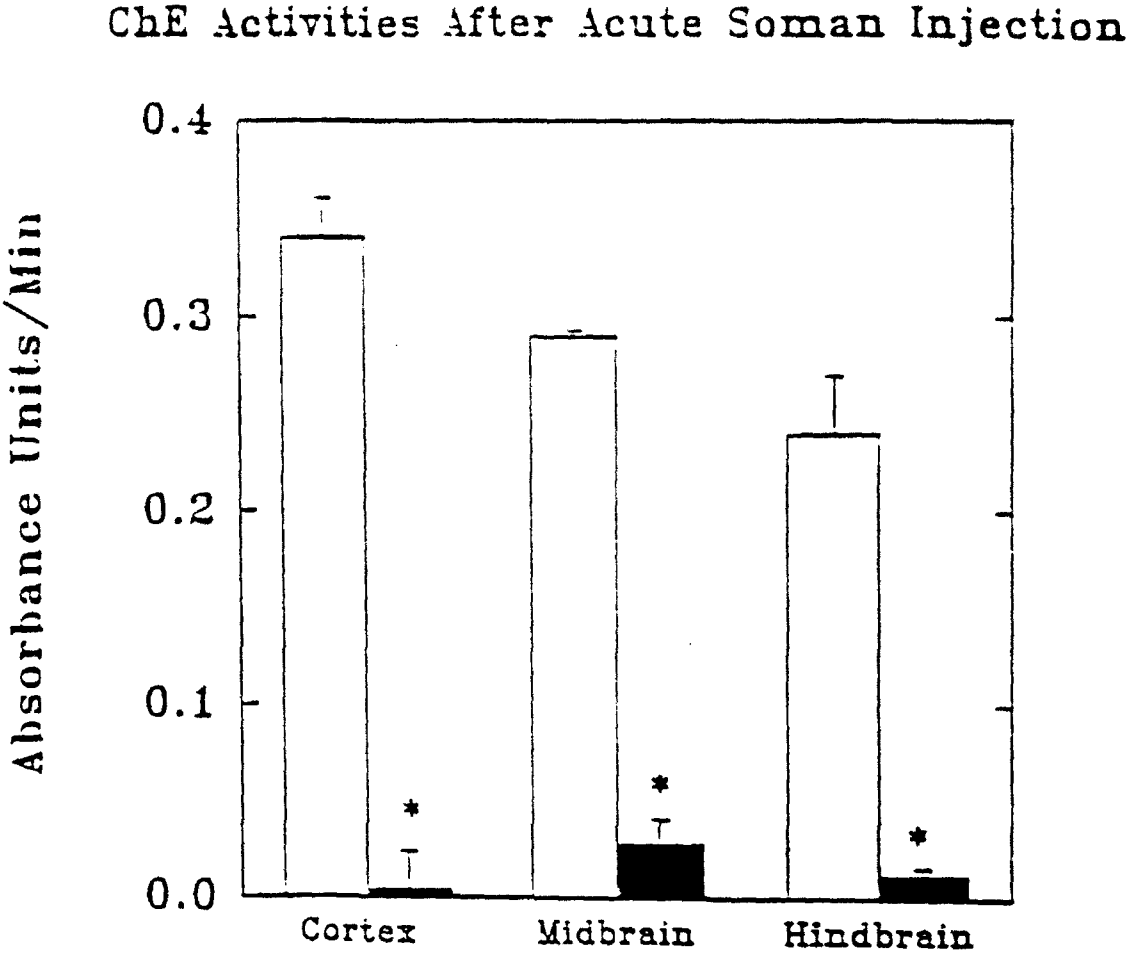


Figure 7

The inhibition of the cholinesterase (ChE) activity in the cortex, midbrain, and hindbrain following an acute soman (70 μg/kg, s.c.) injection in rats. Open bars indicate control saline injected animals. Solid bars indicate soman-injected animals. Error bars refer to the S.E.M. from three experiments. Soman treatment resulted in 90% or more inhibition of ChE activities in all the brain areas tested ($^{*}p>0.01$).

TABLE 8

THE EFFECT OF ACUTE AND SUB-ACUTE SOMAN ADMINISTRATION ON THE BINDING OF [$^3$H]MS TO RAT BRAIN MEMBRANES.

| | Bmax (pmoles/mg protein) | | | |
|---|---|---|---|---|
| | Cortex | Midbrain | Hindbrain | N |
| Control | 2.98 ± 0.14 | 1.31 ± 0.07 | 0.81 ± 0.05 | 13 |
| Time of Sacrifice | | | | |
| 30 min | 3.12 ± 0.252 | 1.44 ± .023 | 0.79 ± 0.02 | 3 |
| 60 min | 3.00 ± 0.217 | 1.56 ± 0.11 | 0.73 ± 0.04 | 6 |
| 120 min | 2.53 ± 0.14* | 1.21 ± 0.14 | 0.67 ± 0.03* | 5 |
| 24 hrs | 2.80 ± 0.12 | 1.27 ± 0.01 | 0.83 ± 0.03 | 3 |
| Sub-acute[a] | 2.48 ± 0.12* | 1.07 ± 0.06* | 0.67 ± 0.03* | 5 |

Rats were sacrificed at the indicated times after soman (70 µg/kg) injection.
[a] = Soman was administered at a sublethal dose of 20 µg/kg every 15 min for a total of six doses. Rats were sacrificed 15 min after the last dose. This regimen resulted in 40% lethality.
* = Significantly lower than saline control. $p<0.05$.

TABLE 9

ChE ACTIVITY AND mAChR FOLLOWING ACUTE SOMAN TREATMENT IN GUINEA PIGS[a]

| ChE Activity (ABS/min) | | | |
|---|---|---|---|
| | Cortex | Midbrain | Hindbrain |
| Control | 0.209 ± 0.007 | 0.200 ± 0.013 | 0.174 ± 0.017 |
| Soman 35 μg/kg, s.c. | 0.016 ± 0.01* | 0.016 ± 0.01* | 0.012 ± 0.01* |
| % Inhibition | 92.3% | 92.0% | 93.1% |

| mAChR, Binding of $^3$H MS | | | |
|---|---|---|---|
| | Cortex | Midbrain | Hindbrain |
| Control | | | |
| Bmax (pmol/mg protein | 2.00 ± 0.12 | 0.96 ± 0.06 | 0.64 ± 0.06 |
| Kd (nM) | 0.23 ± 0.02 | 0.27 ± 0.03 | 0.25 ± 0.02 |
| Soman 35 μg/kg, s.c. | | | |
| Bmax (pmol/mg protein) | 2.14 ± 0.14 | 0.86 ± 0.02 | 0.58 ± 0.06 |
| Kd (nM) | 0.20 ± 0.05 | 0.25 ± 0.05 | 0.26 ± 0.01 |

Each value represents mean ± S.E.M. derived from three to seven experiments.
* = 0.05 compared to control levels.
[a] = Guinea pigs were killed 30 min after soman injection (35 μg/kg, s.c.).

TABLE 10

EFFECT OF CHRONIC ADMINISTRATION OF SOMAN OR PHYSOSTIGMINE ON CORTICAL MUSCARINIC RECEPTORS AND BRAIN CHOLINESTERASE (ChE) ACTIVITY

| Dose soman (μg/kg/day) | 20 | 30 | 40 |
|---|---|---|---|
| % Inhibition binding | -10.2 | -17.4 | -5.64 |
| % Inhibition ChE | 38.2* | 47.1* | 95.5* |
| N | 5 | 4 | 3 |

Soman was administered once daily at the indicated doses for 7 days. Rats were sacrificed 15 min after the last injection of cholinesterase inhibitor.
* = significantly different from saline-injected controls ($p<0.05$).

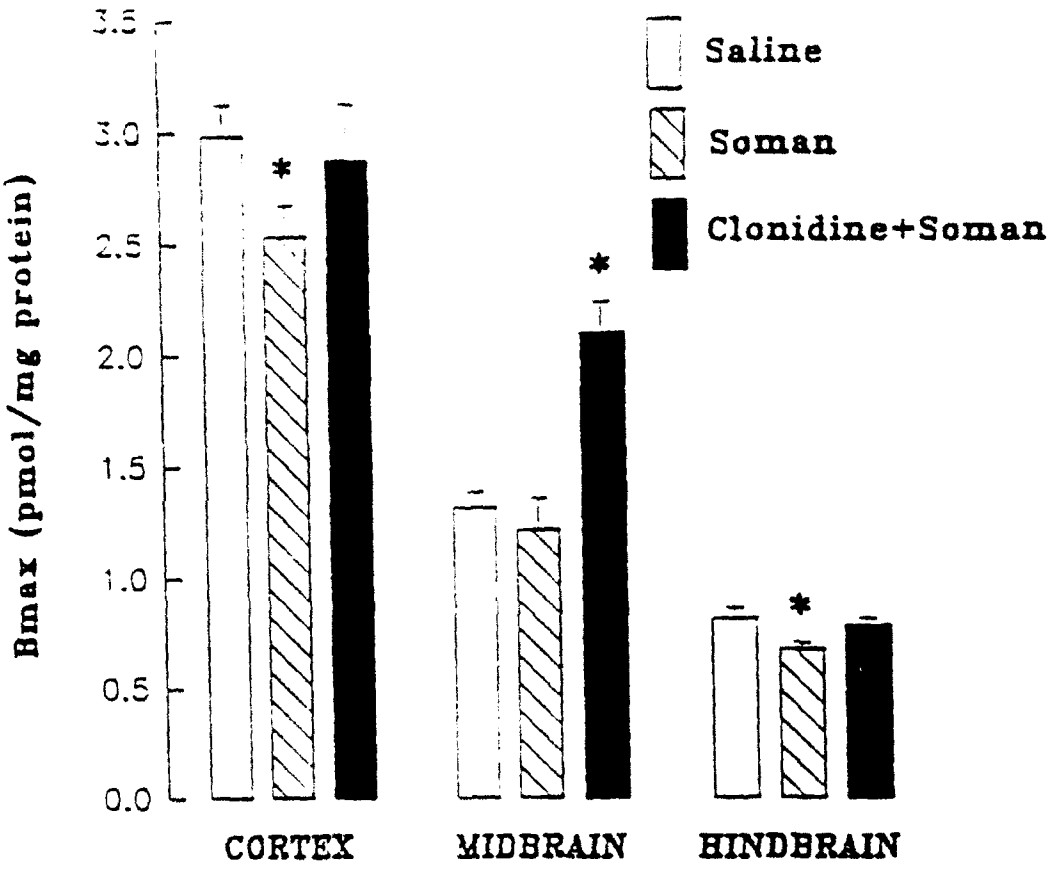


Figure 8

Effect of clonidine pretreatment on the central muscarinic receptor down-regulation produced by acute injection of soman in rats. Open bars indicate data derived from control animals who received two injections of saline spaced 10 min apart prior to sacrifice. Hatched bars refer to animals who received saline 10 min prior to 70 μg/kg of soman. Solid bars refer to animals who received 1 mg/kg of clonidine 10 min prior to 70 μg/kg of soman. Rats were sacrificed 15 min after the last injection. Error bars refer to the S.E.M.

* = significantly different from saline-control mean. $P<0.05$.

## DISCUSSION

### PART 1

The mechanism of the protective actions of clonidine has been investigated [1,4-7,8,10-12,29] and appears to involve more than one component of the cholinergic system. While clonidine does produce a marked inhibition of acetylcholine synthesis and release at peripheral and central muscarinic synapses [4-6,29], its other actions on the cholinergic system include, a reversible inhibition of acetylcholinesterase and a reversible inhibition of muscarinic receptors [1,7]. It is not yet clear to what degree these three mechanisms each contribute to the ability of clonidine to produce protection against the acute lethal actions of soman. However, several centrally-acting α-adrenergic agonists of different chemical structures share this ability with clonidine, and the relative potency as a protective agent was correlated with its affinity for α-adrenergic binding sites labelled with [$^3$H]clonidine [8] Also, the ability of clonidine to inhibit the biosynthesis of brain acetylcholine is mediated through α-adrenergic receptors [6]. It is this action of clonidine, therefore, which appears to predominate in its ability to protect against the acute toxic actions of soman. All three actions, however, can occur at the protective dose levels employed. Despite this multifaceted action of clonidine, the drug has a rather short half-life in rodents [27]. It is possible that clonidine-induced protection of cholinesterase from irreversible inactivation by soman may provide a more chronic form of protection, that is, protection long after the clonidine is metabolized or excreted. Inhibition of brain cholinesterase might account for more free enzyme in the CNS of survivors [7]. We have also speculated [12] that clonidine might offer long-term protection through its ability to block soman induced convulsions. Although the standard protective regimen (physostigmine+artane) might be expected to offer protection *via* similar mechanisms, this study was designed to determine whether clonidine added to the regimen could offer some additional degree of protection, either from acute or delayed soman toxicity and whether this protection extends to a more sophisticated measure of cognitive function. Also, part of the rationale for including clonidine in the pretreatment regimen is related to its ability to inhibit potential side effects produced by other typical protectants. For example we have demonstrated that clonidine inhibits the expression of atropine induced stereotyped behaviors [37] as well as the toxic behavioral and lethal effects of physostigmine [8].

In the present study clonidine administration prior to the standard regimen did not enhance the number of survivors, but significantly reversed soman induced whole body tremor and reduced soman induced deficits in locomotor activity measured 48 hr following soman administration. It was interesting to observe that soman treatment did not produce muscle fasciculations, a classical sign of peripheral cholinesterase toxicity, in saline pretreated animals, confirming previous observations in mice and rats [1,7]. Thus, central toxicity predominates following soman administration. The fact that muscle fasciculations were noted in animals pretreated with the standard regimen suggests that this may be a result of the physostigmine in the regimen. While high doses of soman (as employed in protected rats) might also be expected to produce muscle fasciculations, our previous experience is not consistent with this possibility [1,7].

At 9 days after soman injection, locomotor activity was at or near control (no soman) levels, even in animals surviving $LD_{50}$ or greater doses. This normal exploratory behavior was confirmed 2 weeks later where rats treated with soman exhibited normal step-through latencies during the training trial of the passive avoidance paradigm. Despite this apparent normal motor behavior, rats treated with soman were deficient during the testing trial. The ability of soman to elicit delayed CNS neurotoxicity has been documented [38,39,41]. Poor performance in the passive avoidance paradigm is generally interpreted to indicate memory impairment. Delayed neurotoxicity to organophosphorus inhibitors has been associated with a loss in brain muscarinic binding [14]. Since centrally acting muscarinic receptor antagonists produce marked impairment in passive avoidance learning [17], loss of muscarinic receptors following soman administration may underlie the learning impairment observed in this study.

In an earlier study [12], clonidine employed as the sole protective pretreatment was demonstrated to reduce soman-induced chronic (24 and 48 hr) behavioral impairment. Likewise, in the present study, the standard regimen with clonidine (but not without clonidine) was capable of inhibiting soman induced locomotor impairment at the 48 hr observation . Also, rats pretreated with only the standard regimen were significantly impaired in the passive avoidance test. When clonidine was added to the regimen, passive avoidance behavior was similar to control animals. While its mechanism of action is not yet clear, clonidine appears to provide some protection against the chronic behavioral effects of soman which is not available using the standard regimen. It is possible, therefore, that clonidine induced protection from soman toxicity involves some mechanism not exploited by either physostigmine or trihexyphenidyl.

PART 2

These data are consistent with the finding that addition of clonidine or one of the analogues to a protective regimen which includes pyridostigmine produces more benefit than using pyridostigmine alone. Since pyridostigmine offers protection primarily against the peripheral manifestations of soman toxicity, the added benefit of clonidine is consistent with a central mode of action for the $\alpha_2$ agonist. It was somewhat surprising that, although all the $\alpha_2$ agonists have similar affinity [9] for central $\alpha_2$-adrenergic receptors (except azepexole which has about a 200 fold less affinity for $\alpha_2$ receptors than the other 4 agents), they were quite dissimilar in their ability to 1) increase the PR above pyridostigmine alone, 2) provide protection from the acute toxic behavioral manifestations of soman and 3) provide protection from the more chronic effects of soman toxicity. For example, while guanabenz offered the best Protective Ratio in combination with pyridostigmine, the agonist was not very effective in preventing acute symptoms, in enhancing performance on chronic testing, and was actually worse than pyridostigmine alone in reversing the fall in body weight. Alternatively, lofexidine, which did not enhance the Protective Ratio, still provided protection against acute symptoms which was greater than pyridostigmine alone. Azepexole, which has the lowest affinity of the agonists for central $\alpha_2$ receptors [9], was least effective as a protective agent. Of the 5 compounds tested thus far, clonidine and guanfacine probably provide the best overall enhancement in protection when combined with pyridostigmine.

We have speculated that the ability of clonidine to offer long-term protection against soman toxicity may be related to its ability to inhibit acetylcholinesterase itself (see Introduction). Inhibition of brain cholinesterase might account for more free enzyme in the CNS of survivors [7]. It is also possible that clonidine might offer long-term protection through its ability to block soman-induced convulsive behaviors [12]. The latter action of clonidine may indeed be more important for its long-term protective action since guanfacine, which also offered significant long-term behavioral protection and reduced chronic body weight loss after soman, was 4 times less effective than clonidine in inhibiting brain cholinesterase activity [9]. In fact, both $\alpha_2$ agonists were effective in blocking overt acute behavioral signs of convulsive activity elicited by soman. While diazepam also inhibited the acute convulsive-like behavior following soman, the animals which appeared to be markedly sedate, were not protected from the acute lethality or long-term behavioral toxicity of soman. Clonidine is not routinely employed as an anticonvulsive agent, however, the drug has been shown to be quite effective in limiting seizure production in the audiogenic [26] and kainic acid [3] animal models of epilepsy. In the latter model, clonidine was even more effective than diazepam.

It is not yet clear whether the ability of centrally-acting $\alpha_2$ agonists to limit convulsive behavior following soman administration is mediated through a distinctly adrenergic mechanism or whether $\alpha_2$-mediated inhibition of cholinergic function is involved. For clonidine, however, our earlier studies confirmed the fact that the drug's ability to protect against soman-induced lethality did not require intact stores of brain catecholamines [11]. Thus, central $\alpha_2$-adrenergic receptors and brain cholinergic neurons may play important roles in the development and/or propagation of seizure activity during inactivation of brain cholinesterase as well as with other experimental models of epilepsy.

PART 3

The results of this study agree with the findings by Aronstam and co-workers [2] in that the acute soman injection produced mAChR down-regulation in mice. Acute injection of soman in rats produced mAChR down-regulation 2 hour after administration both in the cortex and hindbrain. This time course for down-regulation appears to be slower than that observed for mice (30 min). Acute administration of soman repeatedly to rats (see the Method) also reduced mAChR density in the brain when measured at approximately 2 hours from the first injection. In both cases ChE activity was inhibited by at least 90%. Rapid loss of mAChRs in response to agonists has also been demonstrated in cultured heart cells, neuroblastoma cells and cerebellar cells by incubating cells with muscarinic agonists [15,18,19,31]. This phenomena was also observed in *in vivo* studies. The mAChRs in rat diencephalon were decreased at 3 hrs following injection of 5 μg of the muscarinic agonist carbachol into the brain [13]. This short-term mAChR down-regulation induced by muscarinic agonists is reversible and may involve a rapid internalization of receptors. Since the quaternary receptor ligand [$^3$H]MS may be excluded from certain intramembrane or intraorganelle sites, the use of this probe may have had been particularly useful in detecting loss of binding sites from the external neuronal membrane [18].

Subcutaneous injection of soman (0.9 of the $LD_{50}$) has been demonstrated to increase acetylcholine (ACh) levels in the rat cortex, hippocampus, midbrain, brainstem and striatum, from 35.2% to 320% above control. ACh levels peaked within 3 hours and gradually returned to baseline [24,42]. This time course for soman-induced ACh elevation is consistent with the time course for changes in brain mAChRs observed in this study. The return of ACh levels towards pre-soman levels after 3 hr is also consistent with the return of mAChRs to baseline 24 hr after soman. However, acute soman treatment in guinea pigs did not produce any alteration in mAChR binding parameters. It is possible that guinea pig mAChRs are not as sensitive to soman as those in rats. Also, species differences may play a role; such as the presence of carboxylesterases [33,34] which have been reported to contribute to differences in the $LD_{50}$'s for soman in different animal species. Therefore, the rat and guinea pig are less responseive than mouse in terms of soman-induced muscarinic receptor down-regulation. Alternatively, additional compensatory mechanisms (e.g., presynaptic down-regulation) may play a more predominant role in rat and guinea pig. This latter possibility is further suggested by the lack of chronic soman (7 days) to maintain mAChR down-regulation, even though ChE levels were still maximally inhibited. Although the accumulation of brain ACh following the administration of ChEIs is the consequence of inhibition ChE [23,35,40,42,44-46]] , the magnitude of ACh elevation does not seem to be associated with the degree of ChE inhibition [25,35,36,45].

This study did not confirm the findings by Churchill and colleagues [14] concerning the ability of chronic administration of soman in reducing mAChRs in certain brain regions (pyriform cortex) as estimated using an autoradiography technique. One explanation for this difference is that in using large brain regions, we were unable to measure putative long-term changes in receptor function which might be observed is small areas monitored by receptor autoradiographic binding techniques. In their study, however, brain damage (hence muscarinic receptor loss) may have been subsequent to seizure or convulsive activity produced by soman. In our study, overt seizure activity was not that prominant.

Finally, our experiments with clonidine pretreatment confirm our earlier studies in mice regarding the ability of this $\alpha_2$-adrenergic agonist to prevent soman-induced mAChR down-regulation [2]. This ability to block soman-induced down-regulation is most likely related to clonidine's ability to limit the accumulation of ACh after subsequent to inhibition on cholinesterase. Thus, one way to view the protective action of clonidine, is that it enhances cholinergic presynaptic down-regulation, one mechanism the organism itself imploys to limit receptor overstimulation.

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